Effect of Various Substrate Conditions on the Microalgal Production of Lipid

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Effect of Various Substrate Conditions on the Microalgal Production of Lipid

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그리고 기숙사 사감조교로 일하는 동안 언제나 저를 챙겨주셨던 박나영 사감 선생님! 항상 감사합니다.

항상 저를 위해 기도해주시는 남궁 민 루카 주임 신부님, 홍 루피나 수녀님, 이제 예쁜 아이의 엄마, 아빠가 된 윤희 플로라 언니, 은우 아오스딩 오빠, 그리고 원동 크리스토폴 청년회 친구들! 항상 저를 위해 기도해주셔서 감사합니다.

그리고 맏언니라고 잘해주지 못했지만 나를 항상 따라주고 챙겨주는 미현이, 정우, 세훈이, 보람이! 언제나 마음으로 다가와주는 정민이! 항상 고마워! 너희와 함께 하는 순간들이 너무 즐거워. 나의 대학원 생활에 즐거움을 준 여인네들 란이, 단홍이, 유일한 여자 동갑내기 친구 지은이! 너희 덕분에 대학원 생활이 더욱 즐거웠어. 가끔 연락해도 너무 좋은 주현이, 채영이, 주림이, 보영이, 지민이.. 언제나 건강하고, 한 번 또 보자!

마지막으로 저의 든든한 버팀목이 되주시는 사랑하는 나의 가족들! 엄마, 아빠! 항상 저를 믿어주시고 응원해주셔서 감사합니다. 언제나 건강이 좋지 않아 걱정이지만, 앞으로는 건강하고 항상 좋은 일들만 가득할꺼예요. 무뚝뚝한 성격에 표현은 많이 못하지만 항상 부모님을 생각하는 딸이랍니다. 사랑합니다! 외할머니, 삼촌, 주연언니! 항상 응원해주시고 걱정해주셔서 감사합니다. 항상 평화가 가득하길!

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김용림

Yong-Rim Kim

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ABSTRACT

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Globally, our demand for energy is ever increasing while the sources we have depended on the last few centuries have become scarcer and will get depleted in the near future. Developing new strategies for biofuel production as a renewable energy is gaining importance. Biodiesel is a non-toxic alternative fuel that is obtained from renewable sources. Microalgae as a feed stock for biofuels have obvious advantages such as rapid growth rate, high lipid content and also mitigation of CO_2. Moreover, microalgae can efficiently uptake nitrogen and phosphorus, which offers an economic and eco-friendly approach of using wastewaters for microalgal cultivation with simultaneous removal of inorganic nutrients.

Various factors such as nutrients and salinity affect the biochemical properties of algae. Algal cultures become depleted of nutrients, when they reach stationary stages of their growth, and an increase in the total lipid is observed at this stage.

In the present study, effect of various micronutrients (Co, Mn, Zn and Cu) on biomass production and lipid productivity of *Micractinium pusillum* cultivated in the Bold Basal Media (BBM) was investigated. In the BBM control, *M. pusillum* showed a biomass production and lipid productivity of 0.79±0.05 and 0.32±0.02 g/L, respectively. The biomass production increased to 1.25±0.01 and 1.28±0.01 g

/L while the lipid productivity to 0.45±0.04 and 0.47±0.05 g/L, when cultivated in BBM with 4X Mn and Cu.

The effect of salinity (NaCl) on the nutrient removal, algal growth, lipid productivity of *Chlamydomonas mexicana* cultivated in municipal wastewater was also studied. Total Nitrogen was completely removed by the addition of 200-400 mmol/L NaCl. Phosphorus removal increased from 77 to 84% as the concentration of NaCl increased from 100 to 400 mmol/ L. 100 mmol/L concentration of NaCl showed a maximum microalgal biomass production and lipid productivity of 0.67± 0.03 and 0.185±0.03 g/L, respectively.

Key word: Microalgae, Micronutrients, Biomass, Municipal Wastewater, Lipid, Nutrients removal

CHAPTER 1

Introduction

Fossil fuel depletion has become a great concern as the world population expands and the demand for basic human needs increase rapidly [1]. A solution to this problem may lie in the renewable energy production. Biofuel production from photosynthetic microorganisms can be considered as an effective strategy [2]. In the aspect of alternative fuels, microalgal biomass is a miniature factory that transforms carbon dioxide and light into biomass rich in mineral components during photosynthesis [3].

Microalgae have many advantages as a potential feedstock for fuel production. 1) microalgae have higher biomass production and growth rates, compared to other energy crops [4], 2) microalgae have the potential to absorb CO_2 and other greenhouse gases for photosynthesis, thus reducing the quantity of greenhouse gas emissions released into the atmosphere [5], 3) microalgae can facilate wastewater bioremediation by removal of NH_4^+, NO_3^-, PO_4^{3-} from a variety of wastewater sources (e.g. agricultural runoff, concentrated animal feed operations, and industrial and municipal wastewaters) [6].

Microalgal biomass can be a source of proteins, carbohydrates and lipids. It shows that microalgae can provide several different types of renewable biofuels such as biodiesel, bioethanol, biohydrogen [7, 8].

Biodiesel has received considerable attention in recent years, as it is a biodegradable, renewable, and non-toxic fuel. It contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than normal diesel [9].

The total lipid content of microalgae ranges between 1-70% of the dry cell weight [10]. Lipids, principally triacylglycerol lipids can be separated and isolated from the harvested microalgae and then converted to biodiesel by

transesterification [11]. The biochemical properties of microalgae can be affected by environment conditions [12-14] such as nitrogen and phosphate limitation [15-16], high salinity [17], and some heavy metal stress [18]. Especially, inorganic phosphate and micronutrients substantially affect numerous metabolic pathways of the microorganisms [19].

The objectives of the present study were: 1) evaluate the effect of micronutrients (Zn, Mn, Cu and Co) on the biomass production, lipid content and fatty acid composition of *M. pusillum* cultivated in the BBM. 2) evaluate the effect of salinity on the biomass, lipid content, glycerol production, fatty acid composition and nutrient removal of *C. mexicana* cultivated in municipal wastewater.

CHAPTER 2

Literature Review

2-1 Microalgae

2-1-1 Characteristics

Microalgae are thallophytes (plants lacking roots, stems, and leaves) that have chlorophyll a as their primary photosynthetic pigment and lack a sterile covering of cells around the reproductive cells [20]. While the mechanism of photosynthesis in these microorganisms is similar to that of higher plants, they are generally more efficient converters of solar energy because of their simple cellular structure. In addition, because the cells grow in aqueous suspension, they have more efficient access to water, CO_2 and other nutrients [21]. Depending on the species, algae can be farmed in either freshwater or saline conditions [22]. Microalgae has been estimated that about 200,000-800,000 species [10].

2-1-2 Potential source of biofuel

There are several ways to convert microalgal biomass to energy sources, which can be classified into biochemical conversion, chemical reaction, direct combustion, and thermochemical conversion (Fig. 1) [6].

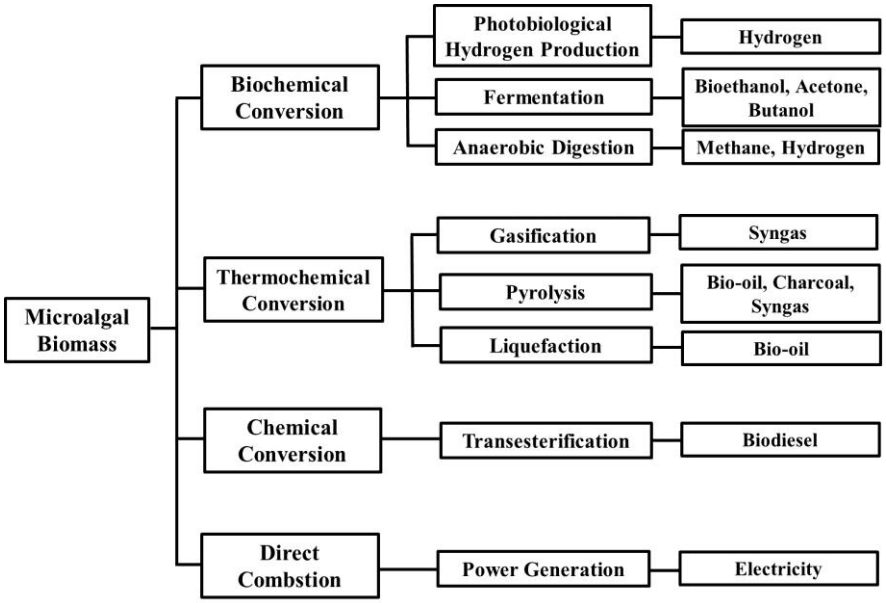


Fig. 1. Conversion processes for biofuel production from microalgal biomass

The utilization of microalgae for biofuels production offers the following advantages over higher plants:

1) rapid growth rate and productivity (microalgae can produce 50 times more biomass compared to higher plants) [23];

2) no competition for land with crops (different types of microalgae are able to grow in a variety of environmental conditions, even on the limited areas of land) [24];

3) the ability to be used in conjunction with wastewater treatment [24];

4) ability to produce non-toxic and biodegradable biofuels as well as high concentrations of commercially valuable compounds such as proteins, carbohydrates, lipids [25];

2-1-3 Cultivation: Factors of cultivation

The chemical composition of microalgae is not an intrinsic constant factor but varies over a wide range, both depending on species and on cultivation conditions.

Numerous factors influence the cultivation of microalgae, including light, nutrient compositions, CO_2, pH, temperature, mixing and salinity [24–26]. The optimal and tolerated ranges tend to be species specific, and may vary according to the desired product. The most optimal parameters as well as the tolerated ranges are species specific and a broad generalization for the most important parameters is given in Table 1 [27].

Table 1. A generalized conditions for culturing microalgae

Parameters	**Range**	**Optimum**
Temperature(℃)	16-27	18-24
Salinity(g/L)	12-40	20-24
Light intensity (Lux)	1,000-10,000 (depends on volume and density)	2,500-5,000
Photoperiod (Light:Dark, hours)	-	16:8(minimum) 24:0(maximum)
pH	7-9	8.2-8.7

- Light

The intensity, wavelength and frequency of light affect the photosynthetic efficiency of microalgae [28]. The light intensity is critical because they grow only when the intensity is higher than the light compensation point. Microalgae and plants have two photosystems: photosystem I with peak absorption at 680 nm and

photosystem II with peaks absorption at 700 nm. The absorptivity of light with different wavelengths vary for microalgae and plants [29]. Light and dark cycle also strongly influence the growth and photosynthetic efficiency of microalgae. It has been suggested that when the frequency of the light/dark cycle increases to higher than 1 Hz, the photosynthetic efficiency is improved [30].

- Nutrients compositions

Many elements have to be provided for the growth of microalgal biomass, such as carbon (C), oxygen (O), hydrogen (H), nitrogen (N), potassium (K), calcium (Ca), Magnesium(Mg), iron(Fe), phosphorus (P), and trace elements. The first three (C,H,O) are obtained from water and air and the latter three have to be absorbed from the culture medium[31]. During cultivation, N and P become limiting. They both are essential element in controlling the growth ratio. On increasing the concentration of N, the growth rate first increased and then decreased, which indicated that a high concentration of N inhibited the growth rate. The metabolic mechanisms of P in the different forms are different in microalgae. Orthophosphate is most easily absorbed and significantly promotes the growth of microalgae [32]. Within a range, the growth rate of microalgae increase with increasing concentration of P, and the opposite occurs when the concentration is too high, which may be due to that the changing N/P inhibits the cell division of microalgae [33].

Several trace elements such as iron, manganese, copper, cobalt, zinc and molybdenum etc., had been observed to had an positive effects on algae growth. The function of this trace elements plays in such process as photosynthesis, respiration, as well as enzyme synthesis [34].

Manganese is necessary in photosynthesis, nitrogen metabolism and to form other compounds required for plant metabolism. Zinc is an essential component of

various enzyme systems for energy production, protein synthesis, and growth regulation. Copper is necessary for carbohydrate and nitrogen metabolism. Copper also is required for lignin synthesis which is needed for cell wall strength. Cobalt functions as a cofactor and activator for enzymes, fixes nitrogen during amino acid production[35].

- CO_2

CO_2 is one of the limiting factors in the photosynthesis. The photosynthesis of microalgae requires a certain CO_2 concentration, and the maximum photosynthetic efficiency is often achieved with CO_2 concentrations from 1 % to 5 % (by volume). Increasing CO_2 levels can improve photosynthetic efficiency, which is consistent with a higher concentration leading to a higher biomass of microalgae [34].

- pH

The pH can also directly affect the permeability of the cell and the hydronium forms of the inorganic salt, and indirectly influence the absorption of the inorgnaic salt. CO_2 is consumed by the microalgae during photosynthesis, thereby increasing the pH of the medium [35]. Therefore, substances like hydrochloric acid and acetic acid have to be added to control the pH to keep the pH from increasing too much to be beyond the tolerance of the microalgae [36].

- Temperature

In general, algal growth increases exponentially with rising temperatures until an optimum level is reached, after which growth declines. Most commonly cultured species of microalgae tolerate temperatures between 16 and 27 ℃ [11].

- Mixing

Mixing is necessary to prevent sedimentation of the algae, to ensure that all cells are equally exposed to the light and nutrients [35].

- Salinity

Salinity is considered as one of the major constraints on species diversity and productivity of natural population of algae [37]. The composition of intracellular lipid of microalgae reported to change in response to environmental salinity [39,40].

2-2 Nutrient removal of microalgae in wastewater

Photosynthetic micro-organisms are useful in bioremediation applications [38]. Effluent produced from the secondary treatment plant contains more amounts of nutrients (nitrogen and phosphorus) and if these effluents are discharged into the ecosystem, it causes eutrophication [39,40]. As an alternative to remove these nutrients, microalgae are suggested to remove the nutrients from wastewater [41]. Microalgae wastewater treatment is ecofriendly and offers the advantage of a cost effective way of nutrient removal and biomass production [42].

2-3 Lipids production of microalgae

2-3-1 Chemical composition of microalgae: Lipids

Microalgae contain lipids and fatty acids as membrane components, storage products, metabolites and sources of energy. The chemical compositions of various microalgae are shown in Table 2 [41,42].

Table 2. Chemical composition of microalgae on a dry matter basis (%)

Strain	Protein	Carbohydrates	Lipids	Nucleic acid
Scenedesmus obliquus	50-56	10-17	12-14	3-6
Chlamydomonas dimorphus	8-18	21-52	16-40	ND
Chlamydomonas rheinhardii	48	17	21	ND
Chlorella vulgaris	51-58	12-17	14-22	4-5
Euglena gracilis	39-61	14-18	14-20	ND
Tetraselmis maculate	52	15	3	ND
Porphyridium cruentum	28-39	40-57	9-14	ND

The total lipid contents of microalgae varied from 1 to 70% of the dry cell weight [43]. Microalgal strains with high lipid content are of great interest in the search for a sustainable feedstock for the production of biodiesel [11].

In view of Table 3 and 4, microalgae appear to be the suitable source of biodiesel that has the potential to completely displace fossil diesel. Unlike other oil crops, microalgae grow extremely rapidly and many are exceedingly rich in oil [44].

Table 3. Comparison of some sources of biodiesel

Crop	Oil yield (L/ha)	Land area needed (M ha)	Percent of existing US cropping area [a]
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae [b]	136,900	2	1.1
Microalgae [c]	58,700	4.5	2.5

[a] For meeting 50% of all transport fuel needs of the United States.

[b] 70% oil(by wt) in biomass.

[c] 30% oil(by wt) in biomass

Table 4. Oil content of some microalgae

Microalga	Oil content (% dry wt)
Botryococcus braunii	25-75
Chlorella sp.	28-32
Cylindrotheca sp	16-37
Monallanthus salina	> 20
Nannochloris sp	20-35
Nitzschia sp	45-47
Schizochytrium sp.	50-77

The lipid in microalgae composed more than 70 % TAG (triglycerols, Fig. 2.) [45].

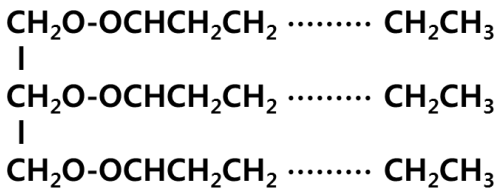


Fig. 2. Molecular structure of TAG (triglycerols)

In general, TAG is a glycerol esterified with three fatty acids and in the presents of alcohol it reacts to form biodiesel with glycerol as a by-product [46].

2-3-2 The change of lipid contents of microalgae

The biochemical composition of algae varies with species, light, temperature, and growth stage. Variation in biochemical composition due to growth stage is frequently related to culture age and nutrient depletion, particularly if an organism is grown in batch culture [47]. Typically, algal cultures become depleted in nutrients, as they enter stationary stages of growth, and total lipid and CHO increase while protein declines [48]. Changes in lipid classes also have been observed as a function of growth stage. In general, phospholipids and glycolipids decline and triacylglycerol and free fatty acids increase [49]. In general, nutrient deprivation can lead to an increase in lipid content, but not for all species of microalgae. On the other hand, nutrient limitation (nitrogen or Si) limitation had less or no significant effect on lipid content of microalgae *Amphora* and *Cyclotella,* respectively [50]. Other studies found that P deprivation could have a positive effect on lipid content [51]. Finally, an osmotic shock might also stimulate the lipids production. When the sodium chloride (NaCl) concentration enhanced, lipid production increase from 60 to 67% (g lipid/g dry weight) [52].

CHAPTER 3

Material and Methods

3-A-1 Algal strain and medium

The *M. pusillum* YSW07 used in this study was isolated from the effluent of a municipal wastewater treatment plant in Wonju, South Korea. The microalgae was cultivated in the Bold Basal Medium (BBM) containing KH_2PO_4 (175 mg/L); $CaCl_2 \cdot 2H_2O$ (25 mg/L); $MgSO_4 \cdot 7H_2O$ (75 mg/L); $NaNO_3$ (500 mg/L); K_2HPO_4 (75 mg/L); NaCl (25 mg/L); H_3BO_3 (11.42 mg/L); trace metal solution (1 mL/L) consisting of $ZnSO_4 \cdot 7H_2O$ (8.82 g/L), $MnCl_2 \cdot 4H_2O$ (1.44 g/L), MoO_3 (0.71 g/L), $CuSO_4 \cdot 5H_2O$ (1.57 g/L), and $Co(NO_3)_2 \cdot 6H_2O$ (0.59 g/L); alkaline EDTA stock solution (1 mL/L) consisting of Na_2EDTA (50 g/L) and KOH (3.1 g/L); and acidified iron stock solution (1 mL/L) consisting of $FeSO_4$ (4.98 g/L) and H_2SO_4 (1 mL) [53]. Regular BBM was altered with different concentrations of Zinc (as $ZnSO_4$), Manganese (as $MnCl_2$), copper (as $CuSO_4$), or cobalt (as $Co(NO_3)_2$). Altered nutrient concentrations are expressed as a fold change compared to the concentration in regular BBM i.e., regular BBM (control) was amended with different concentrations of individual trace metals (Zn, Co, Cu, or Mn), ranging from two- (2X) to six-fold (6X). Separate tests were conducted using micronutrient-depleted media to evaluate the growth and biochemical properties of *M. pusillum* cultivated in growth media lacking a particular nutrient. The pH of media used was adjusted to 6.6 before autoclaving at 121℃ for 15 min.

3-A-2 Microalgal cultivation and growth analysis

Liquid medium (100 mL at 10 % [v/v]) was inoculated with the algal strain in 250 mL Erlenmeyer flasks. The flasks were incubated under white fluorescent light at 40 μmol photon/m^2/s at 27 ℃ for 17 days while shaking at 150 rpm on a rotary shaker (SH-804, Seyoung Scientific, South Korea). Algal growth was measured by determining the optical density of the algal cell suspension at 680 nm using a DR/4000 spectrophotometer (HACH DR/4000, Loveland, USA). The OD_{680} was then converted to a dry weight (DW) concentration using a linear relationship between OD_{680} and dry cell weight (g/L) [54].

$$\text{Dry weight (g/L)} = 0.6857 \times OD_{680} + 0.0041 \ (R^2 = 0.9975)$$

All experiments were carried out in triplicate and the average values are reported.

3-A-3 Lipid extraction and fatty acid analysis

The total lipids were extracted from *M. pusillum* biomass using a slightly modified method of Bligh and Dyer [55]. In brief, cell were harvested and lyophilized. Lipids were extracted with a mixture of chloroform and methanol (1:2, v/v), transferred into a tube, and sonicated for 1hr at maximum power (Power® , Sonic 420, South Korea). The tube was then incubated over night at 27 ℃ with shaking at 100 rpm. An additional aliquot of chloroform (1.25 mL) was added to the tube and the content was sonicated again for 30 min. To separate the chloroform and aqueous methanol layers, 1.25 mL deionized water was added to the tube, which was then centrifuged at 4000 rpm for 10 min. The chloroform layer was collected from the bottom of the tube. A second extraction was performed by adding 2.5 mL chloroform and vortexing. The chloroform layer was gently collected from the bottom of the tube, washed with 5 mL of 5 % NaCl solution, evaporated in a dry oven at 50 ℃. The crude lipid was measured

gravimetrically. Each experiment was carried out in triplicate and average values were reported.

Fatty acids were analyzed using a modification of the method proposed by Lepage and Roy [56]. The crude lipid (~ 10 mg) was dissolved in 2 mL of a freshly prepared chloroform and methanol mixture (2:1, v/v) and transferred to a 10 mL Pyrex tube with a Teflon-sealed screw-cap. 1 mL of chloroform containing an internal standard and transmethylation reagents was added to the tube and mixed for 5 min. The contents were transferred to a 10 mL Pyrex tube, incubated at 100 ℃ for 10 min, cooled to room temperature, and separated into 2 phases by adding 1 mL deionized water. After 10 min of vigorous mixing and centrifugation at 4000 rpm for another 10 min, the chloroform layer was collected from the bottom of the tube using a hypodermic disposable polypropylene syringe and filtered through 0.2 μm syringe filters. Fatty acid methyl esters (FAMEs) in the extracted liquid were quantified by QP2010 Gas Chromatography–Mass Spectrometry (Shimadzu, Japan) with a flame ionization detector using a HP-5MS capillary column. FAMEs were identified by comparing fragmentation patterns with the National Institute of Standards and Technology (NIST) library.

3-B-1 Sampling and characterization of wastewater

Raw municipal wastewater was collected from a primary sedimentation basin at the Wonju wastewater treatment plant in South Korea. Samples were directly filtered through 0.2 μm nylon membrane filters to remove microorganisms and suspended solids. Physicochemical properties of the prefiltered wastewater were analyzed. Total inorganic carbon (TIC) was measured with a Shimadzu TOC-V_{CPH} analyzer. Total nitrogen (TN), total phosphorus (TP), and total chemical oxygen demand (TCOD) were measured using a HACH Kit (HACH DR/4000, Loveland, USA). Chloride (Cl^-), nitrate (NO_3^-), and sulphate (SO_4^{2-}) were determined by single-column ion chromatography (761 compact IC, Metrohm Ltd., Switzerland). Metal ions were analyzed using an ELAN DRC II inductively coupled plasma-mass spectrophotometer (PerkinElmer Sciex, USA). The salinity of the wastewater was adjusted by adding NaCl (up to 400 mmol/L). Wastewater amended with different concentrations of NaCl was used as culture media. The total salinity and conductivity of wastewater were measured using an Orion 115A+ conductivity meter (Thermo Electron Corporation, USA), and the solution pH was measured with a pH meter (Orion 290A). Data were presented as mean ± standard deviation (SD) of triplicate experiments.

3-B-2 Microalga and growth conditions

A microalgal strain *C. mexicana* YSL07 was previously isolated from a freshwater lake at Yonsei University, Wonju, South Korea [7]. This culture was selected for study based on its high growth rate and oil content. Four different culture media with various NaCl concentrations (100, 200, 300, and 400 mmol/L) were prepared using the prefiltered municipal wastewater for cultivation of *C. mexicana*. Prefiltered wastewater without added NaCl was used as a control. The initial optical densities (OD) of the algal suspension were adjusted to an absorbance of 1.5 at 680 nm. 2 mL of algal suspension was used as initial

inoculums. *C. mexicana* was cultivated using 500 mL conical flasks containing 150 mL prefiltered wastewater under white fluorescent light illumination at 45-50 µmol photon m^2/s at 27 ℃ for 10 days while shaking at 150 rpm.

3-B-3 Determination of microalgal biomass and nutrient removal

Total nitrogen (TN) and total phosphorus (TP) were determined at 0 and 10 days of cultivation using a HACH Kit (DR/4000, HACH). Total inorganic carbon (TIC) concentrations were determined at 0 and 10 days using a Shimadzu TOC-VCPH analyzer. Percentages of nutrients removal were calculated by dividing the difference between the initial and final values by the initial value, and then multiplying by 100 [57]. Blank experiments (wastewater without inoculums) were placed in the shaker under the same conditions. Data were presented as mean ± SD of triplicate experiments.

3-B-4 Lipid extraction and fatty acid methyl ester (FAME) composition

Lipids were extracted from the fresh microalga biomass (0.2 g/L) using the methods described by Bligh and Dyer [55]. FAMEs in the extracted liquid were quantified using an Agilent 6890 gas chromatograph (Agilent Technologies, USA) equipped with a flame ionization detector (FID) and a HP-INNO wax capillary column.

3-B-5 Determination of glycerol production

The production of glycerol for osmoregulation of *C. mexicana* under salt stress conditions was identified and quantified using algal biomass. The harvested biomass was washed twice with deionized water. Algal cells were treated with liquid nitrogen, sonicated at high power (Branson Ultrasonic Cleaner 8510, USA) and centrifuged at 3900 rpm for 15 min to remove cell debris [58]. Glycerol

extraction was done twice and the supernatant was pooled. The glycerol content of the cell extracts and glycerol produced in the culture media were enzymatically determined using the glycerol kinase method (KIT method, Cat. F6428, Sigma, USA). Data were presented as mean ± SD of duplicate experiments.

3-B-6 Statistical analysis

One-way analysis of variance (ANOVA) was used to examine the differences among average values. GraphPad Prism version 5.0 for Windows (GraphPad Software, Inc., USA) was used for all statistical analyses, and differences in the variables were considered significant at the $P < 0.05$ level of confidence.

CHAPTER 4

Results and Discussion

4-A-1 Effect of media compositions on the growth rate of *M. pusillum*

Microalgae can grow profusely when supplied with sufficient nutrients under suitable conditions. Algal growth is directly affected by light and nutrient availability, pH and temperature stability, and the initial density of inoculum [59]. Fig. 3-a) shows that depleting individual micronutrients (i.e., Co, Mn, Zn a nd Cu) from the culture media significantly decreased the *M. pusillum* grow th rate. The optical density at 680 nm (OD_{680}) of algae in BBM (control) medium was 0.71±0.02, while for micronutrient-depleted BBM media the OD_{680} ranged from 0.50±0.01 for 0X Cu to 0.59±0.01 for 0X Co after 17 d ays of cultivation. Micronutrients (e.g., Co, Mn, Zn and Cu) are essential f or microalgal growth. These elements play vital roles in the active site of many algal enzymes and are involved in numerous metabolic processes, incl uding photosynthesis and energy storage [60]. Thus, depleting micronutrients from the culture medium adverse affected *M. pusillum* growth. The *M. pusil lum* growth rate increased with elevated nutrient concentrations in the medi um (Fig. 3-b). The OD value of microalgal strain in BBM with double con centration (2X) o-f Mn or Cu reached 0.79 ± 0.01 or 0.75 ± 0.03, respecti vely, after 17 days of incubation, both of which were higher than the contr ol (0.71 ± 0.02). In contrast, increasing the Zn or Co concentration in the growth media had no noticeable effect on growth rate. Based on these resul ts, further experiments evaluated *M. pusillum* growth as a function of Mn o r Cu concentration in BBM. Increasing the Mn or Cu concentration to 4X increased the *M. pusillum* growth (OD_{680} of 0.82 ± 0.01 or 0.87 ± 0.01, res pectively) compared to regular BBM (Fig. 4). Interestingly, increasing the M

n or Cu concentration to 5X or higher had no further effect on the biomass production. These results revealed that a 4-fold increase in Cu or Mn conce ntration maximized the *M. pusillum* growth rate.

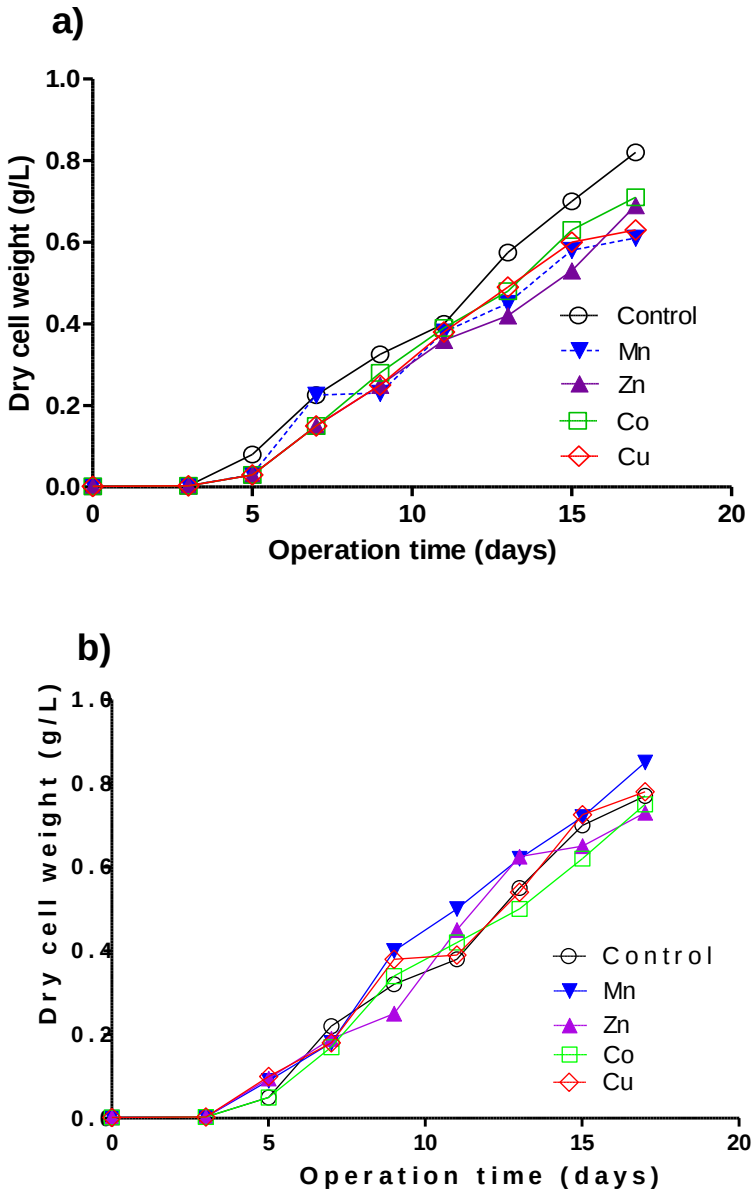


Fig. 3. Dry cell weight of *M. pusillum* grown in BBM a) micronutrient-depleted media (0X) and b) media containing a double each trace metal (2X)

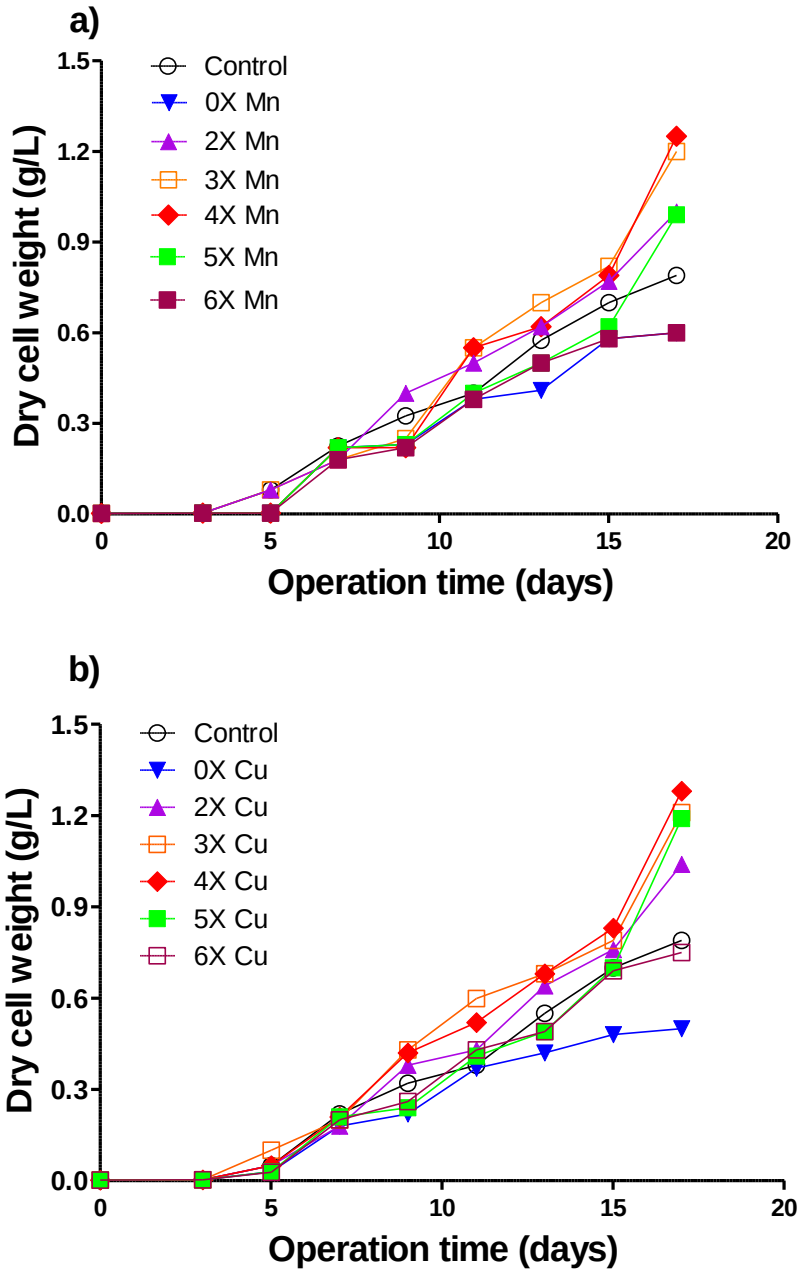


Fig. 4. Dry cell weight of *M. pusillum* grown in BBM with different a) copper and b) manganese concentrations compared with control

4-A-2 Biomass production and lipid productivity

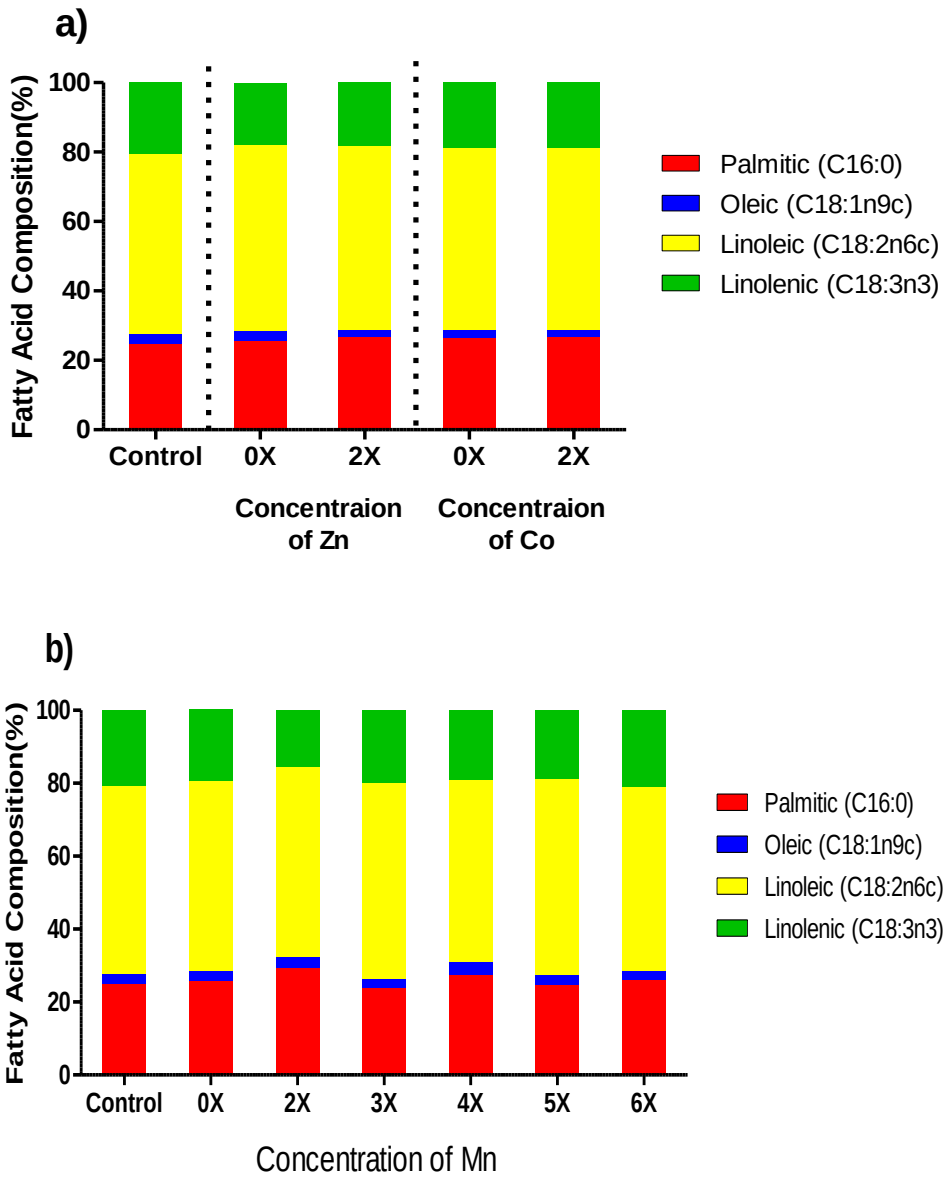
We harvested microalgal cells after the 17 day incubation and examined biomass production and lipid content (Table 5). Depleting Zn, Mn, Co or Cu from growth medium adversely affected algal biomass and lipid production. Increasing the Mn or Cu concentration in the culture media increased algal biomass and lipid production. *M. pusillum* grown in BBM with 4X Cu or Mn produced more biomass (1.25 or 1.28 g/L) and lipids (0.47 or 0.45 g/L) after 17 days of cultivation than the control (Fig.5). The total lipid contents of *M. pusillum* in this study ranged from 31 to 41% of the dry biomass weight. The highest lipid content (41%) was present by the algal strain grown in BBM containing 2X Mn. Many microalgae species can be induced to accumulate substantial quantities of lipids resulting in a high oil yield [61]. Lipid contents of 20-50% of the dry biomass weight have been reported to be quite common [37]. It has also been reported that lipids accounting for more than 90% of the dry biomass of some microalgae have been reported in some culture conditions [2].

Table 5. Effect of trace metals concentration in the growth medium on biomass production, lipid productivity and lipid content of *M. pusillum*

Parameter	Control	Zinc		Cobalt		Manganese					
		0X	2X	0X	2X	0X	2X	3X	4X	5X	6X
Biomass production (g/L)	0.79±0.05	0.69±0.12	0.76±0.04	0.71±0.12	0.85±0.09	0.61±0.12	0.83±0.08	1.20±0.04	1.25±0.01	0.98±0.09	0.99±0.03
Lipid productivity (g/L)	0.32±0.02	0.26±0.01	0.30±0.02	0.24±0.02	0.28±0.01	0.23±0.01	0.34±0.02	0.40±0.02	0.45±0.04	0.39±0.05	0.38±0.04
Lipid content (%)	40±3.1	38±2.5	39±3.5	34±0.5	33±5.9	38±2.9	41±1.5	33±1.8	36±3.1	40±1.5	38±3.4

Parameter	Control	Copper					
		0X	2X	3X	4X	5X	6X
Biomass production (g/L)	0.79±0.05	0.60±0.04	0.80±0.16	1.21±0.12	1.28±0.04	1.19±0.09	0.99 ±0.01
Lipid productivity (g/L)	0.32±0.02	0.24±0.04	0.30±0.16	0.45±0.02	0.47±0.05	0.35±0.02	0.31 ±0.02
Lipid content (%)	40±3.1	40±1.9	38±0.5	37±3.7	38±1.5	32±1.1	31 ±3.5

4-A-3 Fatty acid composition



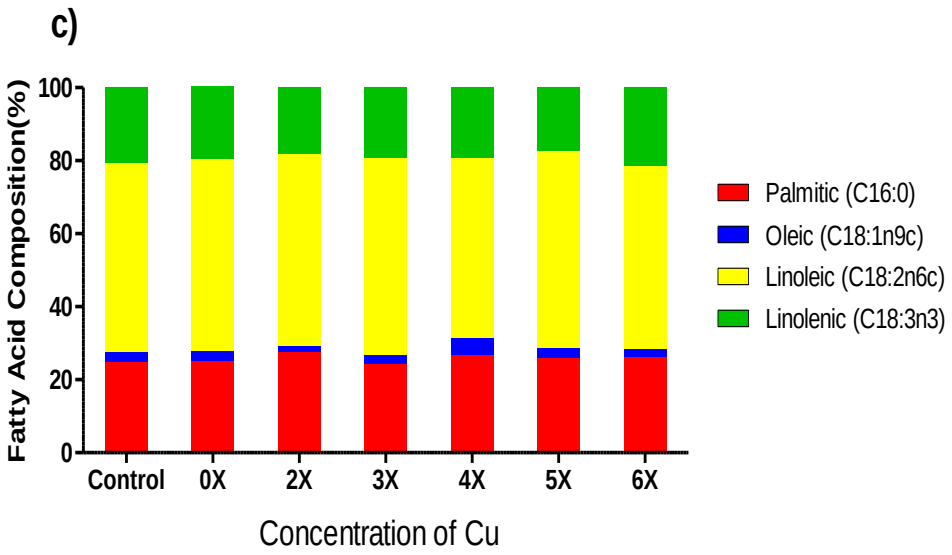


Fig. 5. Effect of trace metals concentration in the growth medium on fatty acid composition (%) of *M. pusillum* a) control, zinc, cobalt , b) manganese, c) copper concentrations

Fig. 5. shows the fatty acid composition in *M. pusillum* harvested from different culture media. Linoleic acid (C18:2n6c) ranged from 49 to 54% of all fatty acids, and was the dominant fraction for all experimental conditions. Linoleic acid was followed by palmitic acid (C16:0) and linolenic acid (C18:3n3) ranging from 24 to 29% and 16 to 22%, respectively. Oleic acid (C18:1n9c) accounted for <5% of all fatty acids. Biodiesel quality depends on the fatty acid composition found 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, and α-18:3 fatty acid components from green algae [62]. A large number of double bonds in a fatty acid makes it more susceptible to oxidation, thus results in economical loss [21]. Nutrient composition of the growth medium, cultivation conditions, and growth phase can readily affect the fatty acid composition in algal biomass [25].

4-B-1 Effect of NaCl on algal growth and biomass, and consequent removal of inorganic constituents from wastewater

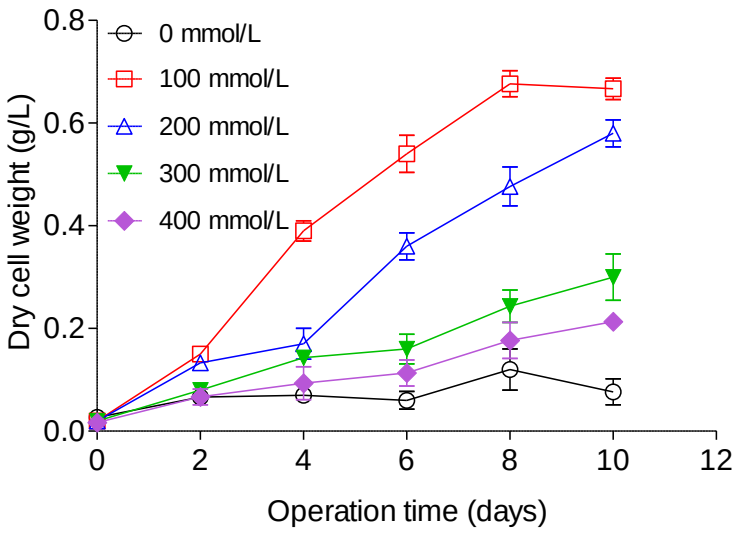


Fig. 6. Effect of NaCl concentrations on the dry biomass of C. mexicana in municipal wastewater

Significantly improved microalgal growth was observed (0.67±0.02 and 0.58±0.03 g/L) after 10 days of incubation in wastewater amended with 100 and 200 mmol/ L NaCl, respectively. When NaCl concentration increased from 300 to 400 mmol/ L, the algal biomass decreased (Fig. 6.). Freshwater microalga *S. obliquus* can grow in synthetic media amended with 600 mmol/L NaCl and the highest dry biomass (0.63 g/L) was obtained at 50 mmol/L after 15 days of cultivation [63]. The absence of plasmolyzed cells suggested that the salt stress of cells under these conditions counterbalanced the drop in the osmotic potential of the surrounding medium [63]. Sodium ion acts through osmolytes that are sequestered in the vacuole to maintain turgor pressure in plants at high salinity [63]. Consequently, algae that have a tolerance for salt stress are able to optimize their growth [64]. Sodium ion is required to facilitate photosynthesis in microalgae through inorganic nutrients uptake, intracellular pH regulation, and alkalotolerance [65,66]. Our result was consistent with earlier reports that the growth of microalgae is substantially inhibited by low or excess salinity due to alterations of basic biosynthetic functions such as photosynthesis and photorespiration [31,63,67]. Microalgae play an important role in biomass production and biological treatment of wastewater [68-70]. The growth of *Chlamydomonas mirabilis* cultivated in sewage mixed with effluents of industrial wastewater was 0.21 g/L with substantial removal of N (from 39.4 to 16.5 mg/L) and P (from 4.65 to 3.05 mg/ L) [68]. The growth of *Chlamydomonas* sp. in industrial wastewater increased [69], and the biomass yield of *Chlamydomonas reinhardtii* was 0.8 g/l/day when cultured in municipal wastewater with 55% removal of N and 15.4% of P [70].

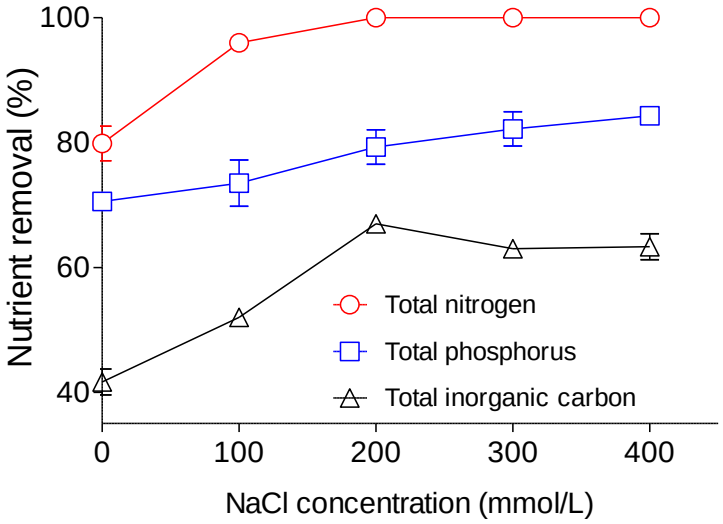


Fig. 7. Effect of NaCl concentration on nutrient removal by *C. mexicana* in municipal wastewater. There was a significant difference among cultures at $P < 0.05$ (TN, $P < 0.0001$; TP, $P < 0.0002$; and TIC, $P < 0.0001$)

Removal of total nitrogen (TN) from wastewater was always higher than total phosphors (TP) removal after 10 days of cultivation regardless of NaCl dose. TN was completely removed with addition of 200-400 mmol/L NaCl (Fig. 7). TP was removed by 68% without NaCl addition, which increased from 77 to 84% with an increase in NaCl from 100 to 400 mmol/L NaCl. The highest removal of TIC (66%) was obtained after adding 200 mmol/L NaCl to wastewater (Fig. 7). In the absence of *C. mexicana* (blank experiment), removals of N (17%), P (14%), and TIC (3%) were obtained after 10 days. Inorganic N/P species are required for growth of all algae [71]. El-Sayed [65] reported that the rise of the N/P removal rate was closely related to the given salinity level and all N/P were uptaken by freshwater green microalga *Scenedesums* sp. which was cultivated for 8 days in growth media with different salinity (up to 100% seawater). Additional N is required for the algal desalination process and enhanced growth of algae as a result of N deficiency in seawater [65]. Yeesang and Cheirsilp [72] found that the freshwater microalga *Botryococcus* sp. survived under high-salinity and nitrogen-

rich culture conditions. Indeed, P assimilation in algae is markedly influenced by a certain level of pH, Na^+, K^+, Mg^{2+}, and/or various heavy metals [73]. However, Rao et al. [17] concluded that the decrease in phosphate was due to the utilization by freshwater microalga *Botryococcus braunii*, which was not influenced by salinity concentration.

4-B-2 Salinity impacts on the biochemical characteristics of *C. mexicana*

4-B-2-1 Effect of NaCl on lipid content

Table 6. Physicochemical characteristics of municipal wastewater

Parameter	Value
pH	8.0±0.1
Total nitrogen (TN, mg/L)	18 ±0.3
Total phosphorus (TP, mg/L)	1.4±0.1
Total inorganic carbon (TIC, mg/ L)	33.4±0.3
Total chemical oxygen demand (TCOD, mg/ L)	63±0.9
Chloride (mg /L)	68±0.2
Nitrate-nitrogen (NO_3-N, mg/ L)	7.3±0.1
Sulfate (mg/ L)	30±1
Calcium (mg/ L)	29±0.3
Sodium (mg/ L)	23±0.2
Potassium (mg/ L)	8.8±0.1
Magnesium (mg/ L)	4.0±0.1
Boron (mg/ L)	0.4±0.1

Concention of total iron, manganese, and barium accounted for ≤ 0.1 mg/L, and zinc, aluminum, and copper were also insignificant (≤ 0.04 mg/L).

Lipids from microalgae are mainly composed of ethyl esters of glycerol and fatty acids, which are suitable for producing biodiesel [73]. Fatty acid profiles and

lipid contents have been used as potential indicators of biodiesel productivity [74]. Table 8 indicates that the amount of lipids accumulated in *C. mexicana* was dependent on the concentration of NaCl in municipal wastewater, which coincided with the previously reported works using synthetic growth media [17]. There was significant correlation ($P < 0.0119$) between NaCl concentration and lipid content (Table 8). Lipid content in the algal biomass increased from 17 to 38% as the concentration of NaCl increased from 0 to 400 mmol/L. Under unfavorable environments many algae species synthesize a large amount of storage lipids such as triacylglycerols [17]. Lipid content was not correlated with the growth of microalga. High lipid content was seen in alga cultivated at high concentrations of NaCl, which was possibly due to salt stress and nitrogen deficiency in the cultivated alga [43]. TN was completely removed by adding 200-400 mmol/L NaCl to wastewater (Fig. 7). Our observation was in good agreement with previous studies showing the effect of salinity on lipid content for different microalgae cultivated in synthetic media [63]. Jiang et al. [75] found that the lipid content (28%) of marine microalga (*Nannochloropsis* sp.) enhanced when the alga was cultured in 50% seawater/wastewater, while the lipid content (23%) of the same alga decreased after it was cultivated in synthetic media for 7 days. Kong et al. [70] reported that the lipid content of *C. reinhardtii* increased up to 26% when it was cultivated in wastewater for 10 days. However, lipid content decreased to 17 % after *C. reinhardtii* was grown in artificial media. In this study, the lipid content of *C. mexicana* was 17 % after cultivation in wastewater for 10 days without adding salt and increased up to 28-38% due to the amendment with 100-400 mmol//L NaCl (Table 9). Outstanding survivability of freshwater microalgae has been reported even when cultivated in highly saline growth media [11]. Zhila et al. [76] demonstrated that a growth medium with high salinity increased microalgae cellular content by improving the formation of TAG.

Table 7. Comparison of salinity effects on lipid content for different microalgal species cultivated in municipal wastewater and synthetic media

Strain	Salinity (g/L)	Medium	Lipid (%)	Reference
Chlamydomonas mexicana[a]	0.2 (0.5)	Municipal wastewater	17	This study
	6.1(11)		28	
	11.8 (20)		29	
	19.7 (32)		33	
	25.1 (41)		38	
Chlamydomonas reinhardtii[a]	-	Artificial medium	17	37
	-	Wastewater	26	
Scenedesmus obliquus[a]	5.8	Chu 13 medium	15	25
	11.6		22	
	17.5		36	
	10		29	
Nannochloropsis oculata[b]	15	F2 medium	31	44
	20		33	
	25		35	
Nannochloropsis sp.[b]	-	F2 medium	23	45
	-	Seawater/wastewater (1:1)	28	

[a] Freshwater microalgae

[b] Marine microalgae

Value sin parentheses represent the conductivity (mS/cm) of wastewater.

4-B-2-2 Effect of NaCl on fatty acid composition

Table 8. Effects of NaCl concentrations in municipal wastewater on fatty acid composition of *C. mexicana* and results of one-way ANOVA analysis (*F-ratios*)

Fatty acid	NaCl (mmol/L)					
	0	**100**	**200**	**300**	**400**	***F-ratio***
	Fatty acid composition (wt %)					
C16:0	28.7±0.7	27.7±0.1	29.2±0.2	29.4±0.4	28.2±0.0	6.01[a]
C17:1	11.3±0.3	13.6±1.1	15.8±0.1	16.3±0.6	17.2±0.2	33.2[b]
C18:1n9c	6.2±0.8	5.6±0.4	5.1±0.1	4.1±0.7	3.8±0.3	7.3[a]
C18:2n6t	18.6±0.5	18.2±0.0	17.1±0.1	17.1±0.1	16.5±0.1	26.9[b]
C18:3n6	29.8±0.1	29±0.1	27±0.2	27.4±1	28.5±0.4	11.9[c]
Other	5.4±0.7	5.9±0.6	5.7±0.4	5.8±0.6	5.8±0.2	-
Total (%)	100±3.1	100±2.3	100±1.1	100±3.5	100±1.3	-

[a]$P < 0.003$, [b]$P < 0.001$, and [c]$P < 0.009$

Salinity fluctuation leads to variations in the fatty acid profiles of algae, which can influence the functions of algal cell membranes and metabolic processes [18]. The degree of saturation of membrane fatty acids is an important parameter in the adaptation of algae to environmental conditions [76]. The composition of fatty acids in *C. mexicana* in which the percentages of palmitic (C16:0), heptadecenoic (C17:1), oleic (C18:1n9c), linolelaidic (C18:2n6t), and γ-linolenic acids (C18:3n6) are plotted as a function of NaCl dose in Table 3,. Under our experimental conditions, the influence of salinity was important and statistically significant and variations in the fatty acid composition of the alga (*C. mexicana*) were mainly due to the salinity of the medium (Table 10). Both C16:0 and C18:3n6 were major forms, accounting for 27-29% and 27-30% of total fatty acid, respectively, under the examined NaCl levels (0-400 mmol/L). C18:2n6t (18±1%) was the next most abundant and the amount of C17:1 was similar to that of

C18:2n6t after adding 200-400 mmol/L NaCl. C18:1n9c ranged from 4 to 6%. Saturated fatty acid (palmitic acid) accounted for 27±2% of total FAME regardless of NaCl concentration, while unsaturated fatty acid (heptadecenoic acid) increased from 11 to 17% as the concentration of NaCl increased from 0 to 400 mmol/L, which was the most remarkable variation in the composition of fatty acids. The fatty acid profile generally depends on the physiological state of algae and the level of salinity [76]. Changes in the fatty acid profile in response to elevated salinity of the medium are inevitable to keep the membrane fluid and prevent its destruction [76]. The other fatty acids accounted for approximately 5±1% in all experimental variations (Table 10).

Under salt stress conditions, unsaturated fatty acids accounted for approximately 70% of total fatty acids. Our observation was consistent with previously reported work in which the accumulation of palmitic and oleic acids in a freshwater microalgal strain (*Botryococcus braunii*) improved with stepped NaCl doses to the growth medium [22]. Rao et al. [22] also found that *B. braunii* changed its fatty acid profiles in response to elevated salinity to retain membrane fluid and prevent its destruction.

A review of literature reveals that saturated acid components in algal biomass are in the range of 25-45%, while unsaturated fatty acids account for 50 - 55 % of total fatty acids [77]. Thus, the ratio of unsaturated to saturated fatty acids in algae oils ranges between 1 and 2, which is somewhat similar to that of plant oils (such as palm) [23]. The quality of biodiesel produced from algae biomass can be expected to be highly competitive with that produced from palm oil. Algal FAMEs were composed predominantly of unsaturated fats, indicating that algae biomass may be preferable for cold weather use due to a typically lower gel point [78].

4-B-2-3 Synthesis of glycerol for osmoregulation in *C. mexicana*

Table 9. Salinity effects on biomass production, lipid productivity, total fatty acid, and glycerol produciton of *C. mexicana* cultivated in municipal wastewater

NaCl (mmol/L)	Biomass production (g/L)	Lipid content (%)	Lipid productivity (g/L)	Total fatty acid (mg/L)	Glycerol production (g/L)
0	0.08±0.02	17±0.7	0.014±0.01	174±6	20±3
100	0.67±0.03	28±0.9	0.185±0.03	301±2	88±9
200	0.58±0.03	29±0.5	0.168±0.04	307±3	100±1
300	0.30±0.04	33±0.9	0.099±0.04	319±5	110±6
400	0.21±0.02	38±0.5	0.079±0.01	346±3	107±4

Adaptation to salt stress in algae is associated with metabolic adjustments that lead to the accumulation of several organic solutes and osmolytes [50]. Compatible osmolytes such as proline, sugars, polyols, amino acids, and glycerol are synthesized in response to salt stress [64]. In this study, glycerol content rapidly increased upon adding NaCl up to 100 mmol/L, but an insignificant further increase was found when the concentration of NaCl in the culture media increased to 400 mmol/L (Table 11).

Gustavs et al. [80] suggested that organisms growing in habitats with high osmotic stress tend to accumulate energetically cheaper organic osmolytes. Metabolic accumulation of glycerol in eukaryotic algae with elevated salinity was described as an osmoregulation mechanism [78]. Algal cells have developed many adaptive strategies in response to different abiotic stresses (such as salinity, dehydration, cold, and excessive osmotic pressure) making use of different mechanisms including changes in morphological/developmental patterns as well as physiological/biochemical processes [79]. Our observations showed that glycerol was not released into the medium. This suggests that most of the glycerol produced and retained in the algal biomass is for the purpose of maintaining

osmotic balance in cells in order to form TAG, resulting in increased lipid production [76]. Lipid content in algal biomass is strongly dependent on salinity in the culture media due to the accumulation of small organic molecules (e.g., glycerol) in response to osmotic pressure [57, 80]. Our results indicate that inorganic carbon was converted to glycerol through either a photosynthetic pathway or metabolic degradation during photosynthesis processes in *C. mexicana*, which resulted in improved lipid production from municipal wastewater with increasing NaCl concentrations (Fig. 7 and Table 9). This interpretation is supported by results from previously reported work [58]. Some freshwater microalgae can be cultivated in high salinity water (i.e., a river mouth where freshwater and seawater meet).

CHAPTER 5

Conclusions

<Part A>

We investigated the effect of culture medium (BBM) supplemented with different concentrations of trace metals on the biomass and lipid productivity of *M. pusillum*. Improvement of both algal biomass growth and lipid productivity was increased the micro-nutrient concentrations such as Cu or Mn in the BBM. It shows that trace metals affect algal biomass and lipid production. *M. pusillum* cultivatied in BBM controled with 4X concentration of Cu or Mn resulted in 1.6 or 1.5-fold increase in biomass yield and 1.4 or 1.3-fold increase in lipid productivity than control, respectively. BBM with 4X concentration of Cu or Mn resulted in 1.6 or 1.5 - fold increase in biomass yield and 1.4 or 1.3 - fold increase in lipid productivity more than the control, respectively.

This study concludes that the concentration of micro-nutrients (Mn and Cu) influences the lipid content and biomass production of *M. pusillum.*

<Part B>

We investigated the growth, nutrient removal, lipid productivity and lipid properties of *C. mexicana* by various concentrations of NaCl in municipal wastewater. The maximum algal biomass yield (0.67 g/L) and lipid productivity (0.185 g/L) were achieved with 100 mmol/L NaCl, respectively. *C. mexicana* removed more than 99 % of the TN and 84 % of the phosphorus with NaCl in municipal wastewater, thus exhibiting excellent nutrient removal efficiency.

These results indicated that, adaptation of *C. mexicana* to salinity was characterized by accumulation lipid.

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국문 요약

다양한 영양염류 조건에 따른 미세조류의 지질 변화

지난 몇 세기 동안 의존했던 화석연료 고갈로 인하여 전 세계적으로 에너지 위기가 도래하고 있다. 이를 해결할 수 있는 대체에너지 개발의 필요성이 대두되고 있으며, 신재생에너지의 한 종류인 바이오연료 생산 연구가 활발하게 진행되고 있다.

바이오디젤은 재생가능한 자원(바이오매스)에서 얻어지며, 독성이 없는 대체 연료이다. 기질로 사용되는 3 세대 바이오매스인 미세조류는 빠른 성장속도, 높은 지질 함량 및 이산화탄소 저감 등의 다양한 장점을 가지고 있다. 또한, 미세 조류는 수중 내 영양염류를 이용하여 성장하며 그 중 질소와 인을 많이 섭취하는 것으로 보고되고 있다. 이는 폐수에 존재하는 질소와 인을 처리하는 경제적, 친환경적 기술로 사용되고 있다.

영양염류의 농도, 염도 등은 미세조류의 성장 및 생화학적 특성에 영향을 미치는 주요 인자들이다. 따라서 본 연구에서는 합성 배양액에 존재하는 미세영양염류(Co, Mn, Zn and Cu) 농도 조건에 따른 미세조류 *Micractinium pusillum* 바이오매스 생산량과 지질 생산량 변화에 대하여 검토하였다. 그 결과, Mn, Cu 농도가 각각 4 배 조건에서 대조군 대비 바이오매스 생산량은 약 1.6 배, 지질 생산량은 약 1.4 배로 증가하여 최대값을 나타냈다. 또한 염도 조건에 따른 폐수에서 배양된 미세조류 *Chlamydomonas mexicana* 의 바이오매스 생산량, 지질 생산량 및 영양염류 제거율 변화에 대해 연구하였다. 연구 결과, 100 mmol/L 염도 조건에서 바이오매스 생산량과 지질 생산량이

최대로 나타났으며, 염도 농도 증가시 총질소(TN)은 99% 이상, 총인(TP)는 84% 이상 제거되어 *C. mexicana* 종은 영양염류 제거에 효과적인 종으로 확인되었다.

핵심되는 말: 미세조류, 미세영양염류, 바이오매스, 폐수, 지질, 영양염류 제거